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Effects of the Transcription Inhibitor Actinomycin D on Postzygotic Development of *Tetrahymena thermophila* Conjugants

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During *Tetrahymena thermophila* conjugation, new somatic macronuclei develop from a common zygotic nucleus derived from meiotic products of the germline, and the old parental somatic nucleus is destroyed. The transcription inhibitor actinomycin D disrupts many events of postzygotic conjugation (cycloheximide causes indistinguishable effects). Early treatment causes a block of all postzygotic development, suggesting a transcription requirement for conjugants to pass a checkpoint, allowing entry into postzygotic development. Thereafter, pair separation, resorption of the old macronucleus, and elimination of one of the new micronuclei are blocked if actinomycin D is added at least 1.5 hr before each of these events normally occurs. Treatment just before DNA rearrangements in the developing macronuclei (anlagen) causes aberrant anlage DNA loss, suggesting that this DNA loss may be caused by inhibition of gene expression involved in genome rearrangements. DNA loss, and correlated lethality, appear to require previous gene expression, since actinomycin D added earlier causes cells to arrest in development without anlage DNA loss, and these conjugants can (at some frequency) complete conjugation and make viable progeny once actinomycin D is removed. The old macronucleus already had been inactivated before most actinomycin D treatments were initiated, indicating that the various induced defects we observed are the result of inhibition of postzygotic gene expression, presumably in anlagen. The defects induced by actinomycin D are similar to defects previously observed in conjugants harboring nullisomic germline deficiencies but proficient old macronuclei.

INTRODUCTION

Tetrahymena thermophila, like other ciliated protozoa, has two distinct nuclei, a diploid germline micronucleus (MIC), and a genetically reorganized somatic macronucleus (MAC). MICs and MACs arise from a common nuclear precursor during sexual development or conjugation. During conjugation (reviewed by Orias, 1986), cell pairing, meiosis of the MIC, and exchange and fusion of haploid meiotic nuclei lead to the formation of a diploid zygotic nucleus. Postzygotic mitoses generate new MICs and the anlagen, or precursors to new MACs. Meanwhile, the old parental MAC condenses (pycnosis), ceases transcription, and is slowly resorbed. During MAC development the germline chromosomes undergo extensive chromosome reorganization, including breakage with telomere formation, breakage and rejoining, and differential amplification (reviewed by Yao, 1989). Two new MICs are formed, one of which is eliminated prior to the first postconjugal cell division (Fig. 1A).

During vegetative proliferation, the MAC is the site of essentially all gene expression, while the MIC is largely inactive (reviewed by Gorovsky, 1980; and Ng, 1986; see also Mayo and Orias, 1981, 1985; Bruns *et al.*, 1983; Kaney and Speare, 1992). However, during MAC development the genetic information derived from the germline MIC becomes active. Genetic analyses suggest that information derived from the developing MAC is required for the successful completion of conjugation. Crossing strains harboring noncomplementing germline nullisomic chromosome deficiencies generate zygotically nullisomic conjugants that arrest during postzygotic development (Davis *et al.*, 1992; Ward *et al.*, 1995). In all nullisomic genotypes tested, pair separation, anlage DNA amplification, and elimination of a new MIC are blocked. In addition, some nullisomic deficiencies block old MAC resorption, and others cause aberrant loss of anlage DNA. Further evidence for essential anlage gene expression comes from the observation that the

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presence of the lethal conjugation mutation *mra* in anlage results in a terminal phenotype indistinguishable from some kinds of nullisomic conjugants (Kaczanowski, 1992). These results demonstrate the insufficiency of the parental MAC to direct postzygotic development and indicate that genes expressed from the anlage, or its precursors, are required for completion of postzygotic events. Although all nuclei descended from the MIC harbor the same noncomplementing chromosome deficiencies as anlagen, nullisomic conjugants show conjugation abnormalities only after the anlage has formed (Ward *et al.*, 1995), suggesting that the anlage is the source of gene expression disrupted in nullisomic conjugants. We undertook the present study to seek independent evidence of anlage gene expression involved in postzygotic conjugation events. The results indicate that postzygotic events require transcription from nuclei present postzygotically (old MAC, new MICs, and anlagen), with the most likely candidate being the MAC anlage.

MATERIALS AND METHODS

Strains

All strains are derived from inbred strain B. Strains UU2 *Pmr/Pmr* (pm-s, IV) and UU5-1 *ChxA2/ChxA2* (cy-s, VI) are heterokaryon strains with MICs that are homozygous for dominant mutations conferring resistance to paromomycin (*Pmr*; Bruns *et al.*, 1985) and cycloheximide (*ChxA2*; Roberts and Orias, 1973; Byrne *et al.*, 1978), respectively. Such heterokaryon strains lack the selectable drug resistance alleles in the MAC and are sensitive to these drugs. Progeny of heterokaryon strains express germline (MIC) drug resistance alleles in their new MACs after conjugation. Therefore, the emergence of drug-resistant cells after conjugation indicates that conjugants have successfully made new MACs (Bruns and Brussard, 1974). Progeny selections were for cycloheximide resistance (*ChxA2*).

Culture Conditions

Growth medium (Neff medium) was 0.25% proteose peptone (Difco), 0.25% yeast extract (Difco), 0.5% glucose (Mallinckrodt), and 0.003% Sequestrene (Geigy). The starvation medium was 10 m*M* Tris–HCl, pH 7.4 (Sigma). Media were sterilized by autoclaving. Cells were cultured at 30°C either in Erlenmeyer flasks as described by Wellnitz and Bruns (1982) or in polystyrene tissue culture flasks. Cell concentrations were determined by optical density.

Matings and Drug Treatments

Cells were prepared for mating as reported by Wellnitz and Bruns (1982) and mated at 200,000 cells/ml in polystyrene tissue culture flasks (ratio of flask volume to solution volume of 10:1). Times given during conjugation refer to the time since the starved parents were mixed together. Matings were done at 30°C.

Actinomycin D was diluted to 100 μg/ml in 10 m*M* Tris–HCl just before use, and an equal volume was added to mating cultures (final concentration, 50 μg/ml) by two different schedules (see Results). This concentration of actinomycin D is sufficient to inhibit transcription in vegetative cells (Ernst and Olenick, 1977) and conjugating cells (Mayo and Orias, 1986). Cycloheximide was used at 25 μg/ml. This concentration is sufficient to kill sensitive conjugants as well as *ChxA2* heterokaryons, because they do not become cycloheximide-resistant before ~30 hr without refeeding (Bruns and Brussard, 1974).

For viability assays, conjugating drug-treated cells were diluted 500-fold into growth medium (Neff) and allowed to recover overnight before selections for progeny were performed.

Cytology

Mating cells were fixed for microscopy in two parts saturated aqueous $HgCl_2$ and one part 95% ethanol, as described by Wenkert and Allis (1984). Nuclei were stained with 5 μg/ml DAPI (4′,6-diamidino-2-phenylindole) in 10 m*M* Tris–HCl, pH 7.4, and observed by UV epifluorescence microscopy.

RESULTS

Cytological Aberrations Induced by Actinomycin D during Postzygotic Development Vary with the Time of Addition

The cytological effects of actinomycin D treatments were quantified in two experiments. Figure 1A summarizes the progress of normal conjugation events in untreated matings in the two experiments. At hourly intervals (beginning at 6 hr and continuing until 16 hr after starting matings) aliquots of mating cells were treated with actinomycin D for 18–30 hr before fixation and DAPI staining. Treatment times in both experiments were sufficient for the majority of cells to reach terminal stages (Fig. 1, legend). Note that untreated cells all finished conjugation by 16 hr (Fig. 1A), and treatment time (18–30 hr) extended well beyond the time conjugation normally is completed. Thus, cells not reaching the final new MAC (NM) stage of conjugation (Fig. 1A) in the presence of actinomycin D are either arrested or greatly delayed in development.

Eight major types of abnormal conjugants were induced sequentially by actinomycin D treatments initiated throughout postzygotic development (summarized by the drawings in panels 1–8, Fig. 1B); a ninth cell type (Fig. 1B, panel 9) is indistinguishable from exconjugants that have successfully completed conjugation and reached the new MAC stage (Fig. 1A). The relative proportions of each type (for each timepoint when actinomycin D treatment was initiated) are shown in the histograms in Fig. 1B (white and black histograms are from experiments 1 and 2, respectively). The stage of development when actinomycin D was

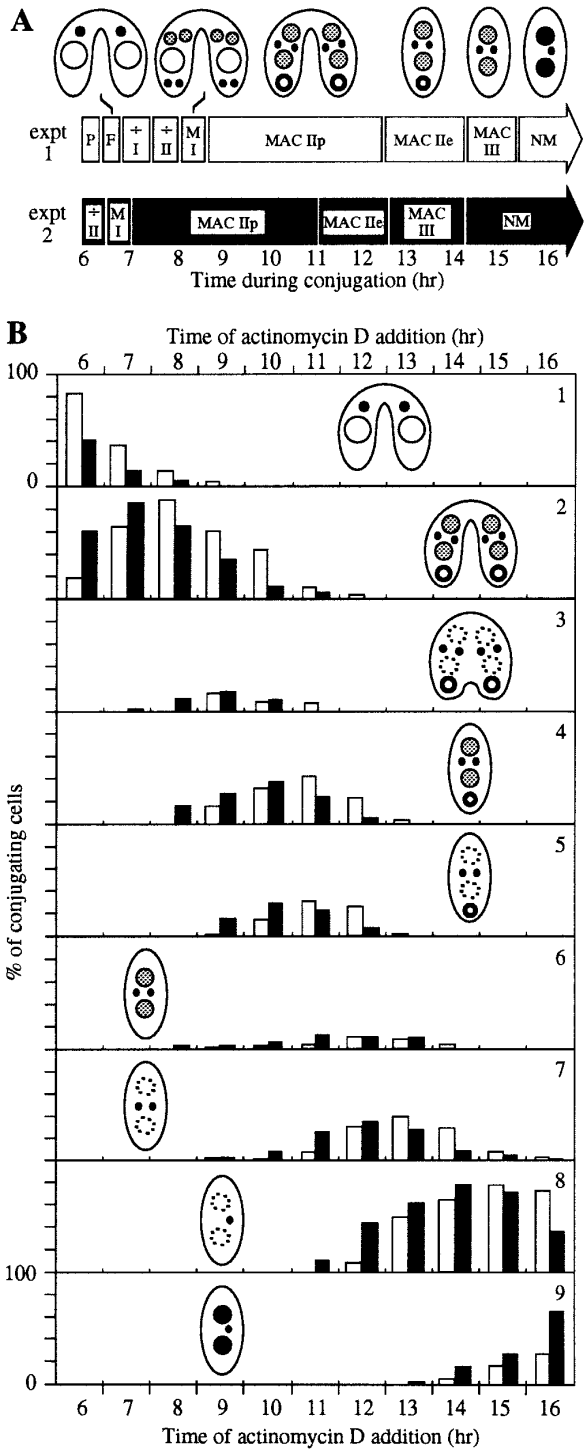


FIG. 1. Effects of actinomycin D on conjugating cells. In two separate experiments, cells were treated with actinomycin D between 6 and 16 hr during conjugation. In experiment 1, aliquots of mating cells from a single mating culture were treated with actinomycin D beginning at hourly intervals between 6 and 16 hr. All cells were fixed for cytological analysis 36 hr after the start of mating. Thus cells were held in actinomycin D for variable times (20–30 hr), depending on when it was added. In experiment 2, 10 separate matings were started at hourly intervals, and actinomycin D was added beginning 6 hr after the last mating was started or 16 hr after the first mating was started. All cells were held in actinomycin D for 18 hr before fixation. The ~1.5-hr difference in the timing of conjugation events between the two experiments is not unexpected and is most likely the result of a delay in the onset of conjugation in experiment 1, since the timing of events in experiment 2 is more typical (Martindale *et al.*, 1982; J.G.W. and G.H., unpublished). (A) Time course of postzygotic development in experiments 1 and 2, determined from cells fixed just before actinomycin D addition. Stages are as defined by Martindale *et al.* (1982) and Harrison and Karrer (1985), and see review by Orias (1986). Following the convention of Martindale *et al.* (1982), each stage is shown to begin when 50% of the pairs have entered that stage. Prezygotic nuclear divisions (P) are followed by fusion of haploid gametic pronuclei (fertilization, F) to form a diploid zygotic nucleus. Determination and differentiation of new macronuclear precursors (anlagen) directly follow two postfertilization nuclear divisions of the zygotic nucleus (÷ I and ÷ II). Macronuclear development is divided into three stages (Martindale *et al.*, 1982; and Harrison and Karrer, 1985). Stage I of MAC development (MI) is distinguished by the centrally located parental MAC (still transcriptionally active), the anterior position of the anlagen, and the posterior location of the new MICs. This is rapidly followed by the MACII stage beginning when the parental MAC condenses (pycnosis) and ceases transcription (Wenkert and Allis, 1984), and the anlagen and new MICs relocate to the center of the cells. We have further subdivided the MACII stage to distinguish cells that are still paired (p) or have separated (e). The MAC III stage begins when the old parental MAC has been resorbed, but before new MIC elimination has occurred. The final new MAC stage (NM) is reached after one of the new MICs is eliminated and the new MAC DNA has undergone amplification. Symbols representing nuclei: micronuclei, small black circles; anlagen, shaded circles; transcriptionally active parental macronuclei, white circles with thin outline; pycnotic old macronuclei, white circles with thick outline; new macronuclei, large black circles. (B) Quantitative summary of conjugation aberrations induced by actinomycin D. For each timepoint of drug addition, cells were scored for the relative proportions of each of the nine observed cytological types (summarized by the drawings in each of the nine panels). This data is summarized by each of the histograms (white and black histograms represent data from experiments 1 and 2, respectively). Horizontal axis, time of actinomycin D addition. Vertical axis for each histogram, percentage of total conjugating cells (0–100%). Approximately 200 conjugants were scored for each sample. Symbols representing nuclei are the same as in A, with the addition of circles with dashed outlines to represent anlagen that have aberrantly lost their DNA. Cytological types 1, 2, 4, 6, and 9 are indistinguishable from fertilization, MACIIp, MACIIe, MACIII, and new MAC stages of normal conjugation, respectively (see above). Cytological types 3, 5, 7, and 8 all show aberrant anlage DNA loss and are often abnormally shaped; type 3 conjugants typically are fused pairs, as described in the text. For conjugants still paired during conjugation, each partner was scored. Sufficient time was given in both experiments for cells to reach terminal stages, as indicated by the fact that treatments of 18 and 30 hr in experiment 2 gave the same relative proportions of each conjugation type (not shown). Samples were scored before noticeable cell lysis, which occurred in some samples if treatment times were extended beyond ~30 hr (30-hr data not shown). Vegetative cells were not scored. Fig. 2 shows photomicrographs of cytological types 2, 3, 7, and 9. No photomicrographic examples are shown for cytological types 1, 4, 5, 6, and 8.

added can be inferred from Fig. 1A [note that the initiation of conjugation in experiment 1 was apparently delayed ~1.5 hr compared to the canonical time course of experiment 2 (Fig. 1, legend), resulting in a corresponding delay in the timing of the effects of actinomycin D; otherwise, the two experimental procedures gave indistinguishable results]. In general, actinomycin D treatment initiated throughout postzygotic development resulted in failure to complete normal events of conjugation (i.e., pair separation, old MAC resorption, and new MIC elimination) and some treatments resulted in aberrant anlage development (Fig. 1B). These conjugation abnormalities are described in greater detail below. None of the conjugation abnormalities induced by actinomycin D were observed in untreated matings, and virtually all untreated cells completed development (Fig. 1A).

When actinomycin D was added just before fertilization (6 hr in experiment 1, Fig. 1A), most conjugants (>80%) did not progress beyond fertilization and subsequently did not complete events leading to MAC development, nor did they undergo pair separation in the presence of actinomycin D (Fig. 1B, graph 1). These conjugants apparently were viable throughout the duration of actinomycin D treatment, since most pairs were observed to swim during treatment (not shown); however, the fate of these conjugants upon removal of actinomycin D was not investigated further. It is possible that these conjugants would complete development once actinomycin D is removed, or maybe they abort further development, separate, and retain their parental MACs as ''nonconjugant'' pairs (Scholnick and Bruns, 1982). The inability of conjugants to progress beyond the fertilization stage is presumably the result of inhibition of gene expression from the parental MAC, since it is still active during this period (Wenkert and Allis, 1984), and anlagen have not yet formed (Fig. 1A, 6 hr in experiment 1).

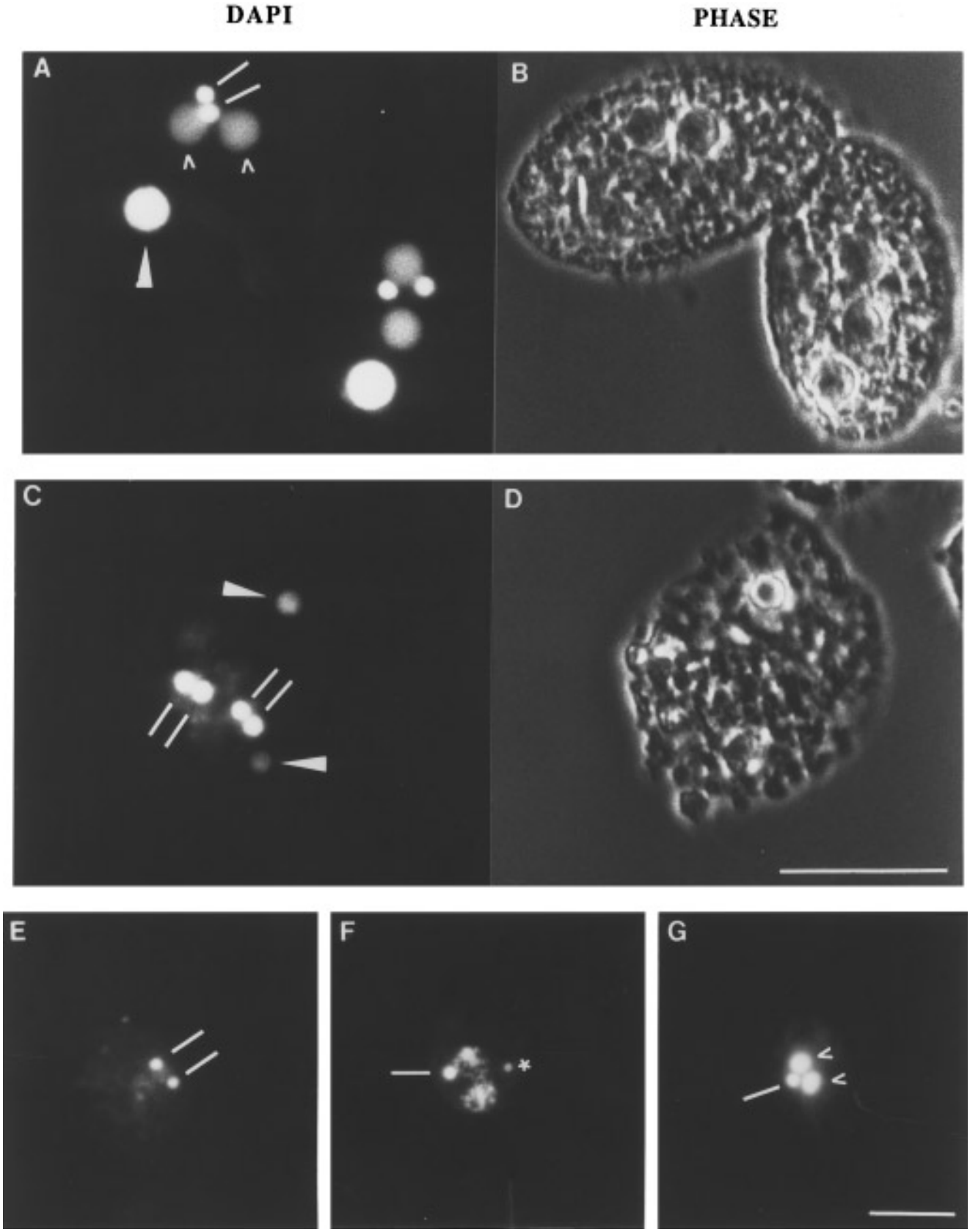
Actinomycin D treatment initiated just following fertilization, when most conjugants were undergoing postzygotic nuclear divisions (7 hr in experiment 1 and 6 hr in experiment 2, Fig. 1A), did not block the majority of pairs from progressing to the MACIIp stage in the presence of actinomycin D (Fig. 1B, graph 2). Note that no cells were observed to be stalled between the fertilization and MACIIp stages; that is, we saw no population of cells that completed the first postzygotic nuclear division but were unable to execute the second nuclear duplication cycle, anlage determination, and relocation and pycnosis of the old parental MAC. This suggests that upon reaching fertilization the subsequent steps leading to the MACIIp stage apparently proceed without a need for ongoing transcription.

Once pairs reach the MACIIp stage the old parental MAC abruptly ceases transcription and its DNA is degraded (Wenkert and Allis, 1984; Davis *et al.*, 1992). Therefore, the effects of actinomycin D treatments on conjugants that have reached the MACIIp stage at the time of drug addition presumably result from the inhibition of anlage gene expression (see Discussion). Addition of actinomycin D during early postzygotic development (6–9 hr, Fig. 1A, experiment 2) inhibited progression beyond the MACIIp stage (Fig. 1B, graph 2). Specifically, pair separation, old MAC resorption, and elimination of one new MIC all were blocked if actinomycin D was added at the onset of postzygotic development. Figures 2A and 2B show an example of such a mating pair treated with actinomycin D beginning at the onset of the MACIIp stage. This pair is indistinguishable from a normal MACIIp pair (Martindale *et al.*, 1982); however, it is greatly delayed in development. Young anlagen have a phase-refractile appearance that normally is lost late in conjugation (Allis and Dennison, 1982); however, note that the anlagen of these actinomycin-treated cells are visible by phase-contrast microscopy (Fig. 2B), indicating that these anlagen had not progressed normally in development in the presence of actinomycin D. This conclusion is supported by the faint DAPI staining of anlagen in such conjugants (Fig. 2A). The faint anlage staining in this conjugant is comparable to the 4C DNA content typical of MACIIp conjugants (Allis and Dennison, 1982; Roth and Cleffmann, 1986) and is visibly less than the staining of new MACs that have completed DNA amplification (compare the new MACs in Fig. 2G to the anlagen in Fig. 2A). This suggests that the DNA amplification that normally occurs in anlagen (Doerder and Debault, 1975; Allis and Dennison, 1982; Roth and Cleffmann, 1986) and that is blocked in nullisomic and *mra* progeny (Ward *et al.*, 1995; Kaczanowski, 1992) is also inhibited by actinomycin D. At least some conjugants blocked at the MACIIp stage by actinomycin D remained viable during this extensive period of actinomycin D treatment (see below).

When actinomycin D was added at progressively later times, pair separation, old MAC resorption, and new MIC elimination did occur in the presence of actinomycin D or already had occurred before drug addition (Fig. 1B, graphs 4–9, graphs 6–9, and graphs 8 and 9, respectively). The ability of conjugants to complete these three events in the presence of actinomycin D is described further below.

Addition of actinomycin D late in the MACIIp stage or in the MACIIe stage (⩾9 hr, Fig. 1A, experiment 2) resulted in aberrant loss of anlage DNA (Fig. 1B, graphs 3, 5, 7, and 8). As described below, inviability of conjugants treated with actinomycin D coincided with the appearance of conjugants that had lost their anlagen. Figure 2C shows an example of a pair treated with actinomycin D beginning at 10 hr into conjugation; four new MICs and two pycnotic old MACs are discernible, but the anlagen that were present when actinomycin D was added at the MACIIp stage (see Fig. 1A, 10 hr in experiment 2) are no longer discernible. Like the conjugant shown in Fig. 2A, the one shown in Fig. 2C failed to complete pair separation, old MAC resorption, and new MIC elimination. Note that this pair had aberrantly fused (Fig. 2D; compare with the normal pair in Fig. 2B). As can be seen in panel 3 of Fig. 1B, the population of cells showing pair fusion represents a relatively small proportion of the total, and fusion only resulted when actinomycin D treatment was initiated ~2–3 hr preceding the time when most pairs would have separated (Fig. 1A).

Figure 2E shows another example of anlage DNA loss, in this case in an exconjugant (actinomycin D added at 12 hr). Two MICs, but not even vestiges of anlagen, are visible.



Anlage DNA loss in exconjugants often is associated with abnormalities in cell shape. Typically, these exconjugant cells look ''swollen'' and are round, not spindle-shaped like normal exconjugants and vegetative cells (not shown). In relatively rare instances cells apparently arrest at intermediate stages of anlage DNA loss; for example, in some exconjugants (Fig. 2F), anlagen have an abnormal punctate appearance and appear to have lost DNA. Perhaps coincidentally, the cell shown in Fig. 2F had apparently arrested in the process of eliminating one of the new MICs (note the weak staining and small size of the nucleus marked with the star). While such occurrences were observed quite infrequently, they do suggest that the process of MIC elimination can be interrupted at intermediate stages by actinomycin D (see Discussion).

Actinomycin D did not affect the morphological appearance of exconjugants if added after most conjugation events had been completed (Fig. 1B, graph 9; see example Fig. 2G). Such conjugants either finished conjugation before actinomycin D addition or were able to complete the final events of conjugation in the presence of actinomycin D. At least some of these normal-looking exconjugants were viable (see below).

Similarity of Cycloheximide and Actinomycin D-Induced Effects

To further investigate whether the aberrations induced by actinomycin D result from inhibition of gene expression, we investigated the effects of the translation inhibitor, cycloheximide. Cycloheximide induced the same spectrum of conjugation abnormalities as actinomycin D: for example, its addition during the early MACIIp stage prevented exit from that stage (i.e., pair separation, old MAC resorption, and new MIC elimination are inhibited), and later treatments resulted in aberrant anlage DNA loss and abnormal cell shape (not shown).

That cycloheximide induces the same spectrum of conjugation abnormalities as actinomycin D strongly indicates that the defects induced by both drugs result from the inhibition of the transcription and translation pathway and not from other unspecified mechanisms. This conclusion is further supported by the marked similarity of actinomycin D- and cycloheximide-induced abnormalities to the genotype-specific effects of various nullisomic chromosome deficiencies (Ward *et al.*, 1995).

Timing of Actinomycin D Inhibition of Pair Separation, Old MAC Resorption, and New MIC Elimination

To better reveal the timing of the inhibitory effects of actinomycin D on the individual processes of pair separation, old MAC resorption, and new MIC elimination, the proportion of all conjugants that completed each of these events was determined for each timepoint of actinomycin D addition (experiment 2), regardless of other aspects of the cell's appearance.

Figure 3A shows the kinetics of pair separation in untreated, control conjugants during conjugation (circles). Fifty percent of the untreated conjugants separated at ~11 hr, as expected (Martindale *et al.*, 1982). Figure 3A also shows the ability of pairs to separate following an 18-hr treatment with actinomycin D (squares) as a function of when actinomycin D was added during the mating; in this case the horizontal axis indicates the time of drug addition. When actinomycin D was added at 9 hr (~2 hr before pair separation in half of the untreated control cells), ~50% of the conjugants were able to separate in the presence of actinomycin D. This suggests that actinomycin D loses its ability to inhibit expression of genes required for pair separation if added later than ~9 hr. If, as observed by Ernst and Olenick (1977) in vegetative cells, transcription inhibition is delayed ~15–30 min after actinomycin D addition, then the lag between gene expression and pair separation may be closer to ~1.5 hr, and the latest gene(s) required for pair separation is expressed before ~9.5 hr. Similar curves are presented for completion of old MAC resorption and new MIC elimination (Figs. 3B and 3C). Again, in each case completion of the process appears to require expression ~1.5–2 hr before the time that the event normally occurs.

Reversible and Irreversible Effects of Actinomycin D on Viability and Anlage Development

The kinetics of aberrant anlage DNA loss in the presence of actinomycin D, as a function of the time of drug addition, are shown in Fig. 4. The fraction of conjugating cells showing anlage DNA loss gradually increased with the time during MAC development when actinomycin D is added and was maximal between ~12 and ~14 hr. This corresponded to the time and stage when anlage DNA rearrangements occur (Yokoyama and Yao, 1982; Austerberry *et al.*, 1984).

The inhibitory effects of actinomycin D are reversible in vegetative cells (Mayo and Orias, 1986; J. Ward, unpublished) and in conjugants treated at early and late times

FIG. 2. Photomicrographs of conjugants treated with actinomycin D during postzygotic development. Epifluorescence micrographs of DAPI-stained cells treated with actinomycin D for 18 hr beginning 7 hr (A), 10 hr (C), 12 hr (E), 14 hr (F), and 16 hr (G) during conjugation. (B and D) Phase-contrast images of A and C, respectively. Straight lines, micronuclei; V, anlagen in A, new macronuclei in G; arrowheads, old macronuclei. As noted in the text, the pair shown in C and D has become fused. (F) The star indicates what is apparently one of the new MICs that was in the process of elimination when the cell was fixed. Scale bar in D also pertains to A–C. Scale bar in G also pertains to E and F. Both scale bars, 20 μm. Conjugants were generated during experiment 2 (see Fig. 1A).

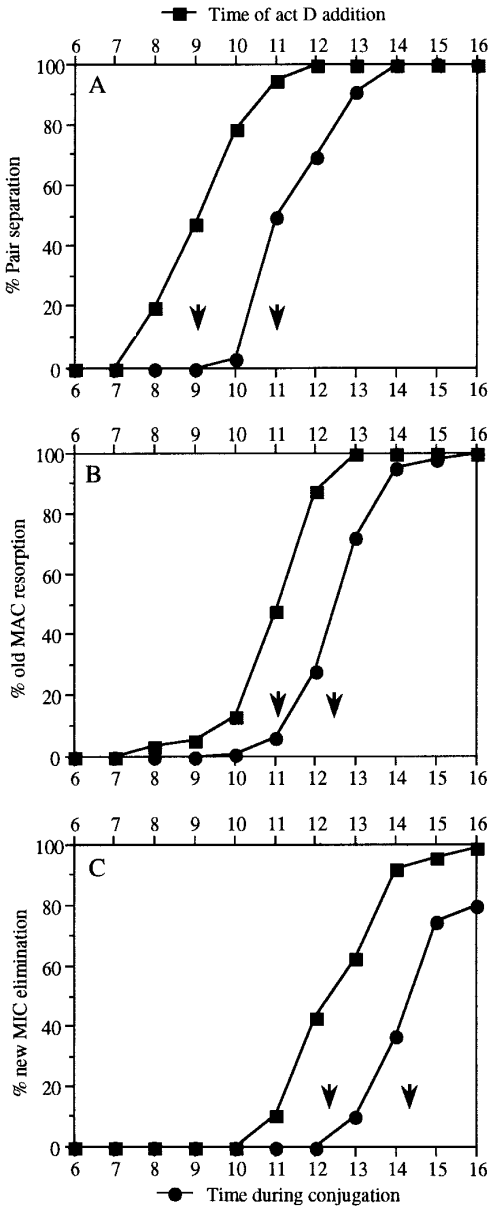


FIG. 3. Time lags in the effects of actinomycin D on pair separation, old MAC resorption, and MIC elimination. Circles, kinetics of (A) pair separation, (B) old MAC resorption, and (C) new MIC elimination in untreated conjugants as a function of time during conjugation (summarized in experiment 2, Fig. 1A). Squares, percentage of conjugants that completed (A) pair separation, (B) old MAC elimination, and (C) MIC elimination after 18-hr treatment with actinomycin D, as a function of the time during conjugation that actinomycin D was added (from Fig. 1B). Horizontal axis, time during conjugation (circles) and time of actinomycin D addition (squares). Vertical axis, percentage of conjugating cells completing each event. Arrows point to times when 50% of the conjugants have accomplished a particular event in the absence of actinomycin (right arrow) or have retained the capacity to accomplish that event in the presence of actinomycin (left arrow). Data are from experiment 2 (see Fig. 1).

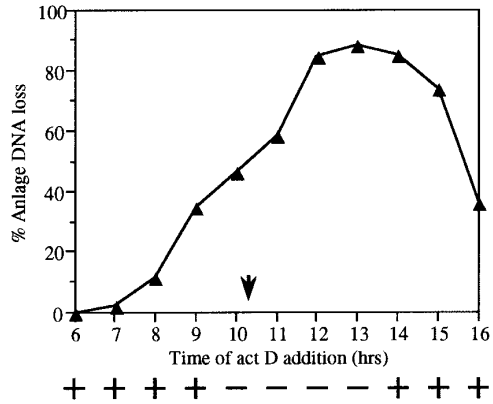


FIG. 4. Correlation between aberrant anlage DNA loss and inviability induced by actinomycin D. The graph shows the percentage of conjugating cells that have aberrantly lost anlage DNA after an 18-hr treatment with actinomycin D, as a function of the time that actinomycin D was added during conjugation (data from experiment 2, Fig. 1B). After an 18-hr treatment with actinomycin D, ~10,000 conjugating cells (based on the initial concentration of cells in the mating culture) from each sample were diluted (~500-fold) away from actinomycin D in growth medium and allowed to recover overnight. These fed cultures were then assayed for the production of viable progeny (see Materials and Methods). The viability results are summarized beneath the graph: (+) viable progeny produced, (−) no viable progeny produced. The arrow points to the time when 50% of the conjugants have lost anlage DNA.

during postzygotic development (see below; Mayo and Orias, 1986). However, the effects of actinomycin D are irreversible when added at intermediate times during postzygotic development: No viable progeny cells (out of ~10,000 conjugating cells) were recovered when actinomycin D was added between 10 and 13 hr (Fig. 4). This coincided with the time when actinomycin D addition caused cells to lose their anlage DNA, as one might expect, since these conjugants have inactivated or already eliminated the old MAC and have destroyed the new MAC.

While conjugants that have completely lost their anlage DNA can account for some of the inviability, some cells with apparently unaffected anlage also must be inviable. For example, no viable progeny were produced from samples treated with actinomycin D beginning at 10 hr, yet ~50% of these conjugants still had anlagen after an 18-hr treatment (Fig. 4). Either these inviable cells with visible anlagen had lost essential but undetected amounts of anlage DNA, or gross DNA loss is a secondary consequence of a general lethality and is seen only in some cells. The latter explanation does not seem likely, since DNA loss was restricted to anlagen (the other nuclei did not show abnormal DNA loss), and earlier treatments with actinomycin D did not result in anlage DNA loss or complete lethality (see below). Furthermore, the observation that anlagen DNA was only partially lost in some cells (Fig. 2F) is consistent with the former explanation that some cells may have suffered undetected DNA loss.

The frequency of conjugants that have lost their anlage DNA decreases in samples treated between 14 and 16 hr, and some viable progeny begin to emerge (Fig. 4). This decrease coincides with the appearance of normal-looking exconjugants that are apparently unaffected by actinomycin D (Fig. 1B, panel 9; and Fig. 2G).

Interestingly, at least some viable progeny emerged from samples treated with actinomycin D before 10 hr (Fig. 4), even though these conjugants apparently did not progress beyond the MACIIp stage in the presence of the drug (Fig. 1B, graph 2; Figs. 2A and 2B). Presumably, some of these conjugants were able to complete conjugation normally after actinomycin D was removed. These results indicate that anlage DNA loss and death, induced by actinomycin D treatments later in MAC development, requires pairs to proceed beyond a certain stage of development and that this step(s) can be reversibly interrupted by actinomycin D early in postzygotic development.

DISCUSSION

Early Events Under the Probable Control of the Parental MAC and a Possible Checkpoint Controlling Entry into Postzygotic Development

When conjugants were treated with actinomycin D prezygotically, they failed to proceed beyond the fertilization stage, and presumably they aborted further development, separated, and retained their parental MACs. This is reminiscent of two alternative conjugation pathways, round I of genomic exclusion (Allen, 1967; Doerder and Shabatura, 1980), and the low-frequency production of ''nonconjugants'' in normal matings (Scholnick and Bruns, 1982). In both pathways, conjugants complete prezygotic events normally, but do not commit to MAC development or old MAC elimination, and produce exconjugants that retain their original parental MACs. Nonconjugants can proceed as far as fertilization, as indicated by the fact that some nonconjugant clones have cross-fertilized MICs (Scholnick and Bruns, 1982). Possibly, conjugants that arrest at fertilization in actinomycin D (Fig. 1B, graph 1) result from the disruption of a normal signal(s) that also fails to occur properly in round I conjugants and nonconjugants. Taken together, these observations suggest that without proper signals, the conjugants do not proceed beyond a checkpoint and to do so requires transcription from the old parental MAC. Recall that once pairs have passed this putative checkpoint they are able to perform the subsequent steps leading to the MAC IIp stage (postzygotic nuclear divisions, anlage determination, pycnosis of the old MAC, and relocation of the postzygotic nuclei) without the apparent need for ongoing transcription. Not too surprisingly, this brief period of insensitivity to actinomycin D coincides with the stage of development when most cells have inactivated the old MAC (or soon will), but have yet to produce a new MAC to take its place. Once anlagen appear, actinomycin D treatment begins to disrupt postzygotic development (see below).

Role of Early Anlage Gene Expression in Postzygotic Development

The various conjugation defects induced by actinomycin D—aberrant anlage DNA loss, blocks to pair separation, old MAC resorption, and new MIC elimination—are all defects seen in conjugants nullisomic for germline chromosomes (Davis *et al.*, 1992; Ward *et al.*, 1995). The marked similarity between defects seen in nullisomic conjugants and those induced by actinomycin D indicates that in both cases the same processes are being disrupted. Phenotypes of various zygotic nullisomic deficiencies led us (Ward *et al.*, 1995) to conclude that postzygotic conjugation events are controlled by at least three independent pathways, one involving anlage development, another determining resorption of the old MAC, and one or more pathways determining pair separation and new MIC elimination. Actinomycin D treatments confirm that anlage DNA fate is independent of the other three processes. Thus, separation occurs irrespective of anlage DNA loss (Fig. 1B, types 4 versus 5), as does old MAC resorption (Fig. 1B, types 6 versus 7) and new MIC elimination (Fig. 1B, types 8 versus 9).

While nullisomic conjugants have euploid parental MACs, they are deficient in the germline MIC, and consequently both prezygotic and postzygotic nuclei (anlage and new MICs) derived from the MICs are deficient for these regions of the germline. Therefore, it is possible, in principle, that any one or more of these nuclei could be sources of gene expression blocked by nullisomic deficiencies. Indeed, transcripts accumulate in premeiotic MICs (Sugai and Hiwatashi, 1970; Martindale *et al.*, 1985), so some genes involved in postzygotic development could, in principle, be transcribed from premeiotic MICs. However, actinomycin D treatments initiated during postzygotic development produced defects in each of the developmental processes affected in nullisomic conjugants. This indicates that at least one gene product required in each of these processes is expressed postzygotically.

Three types of nuclei are present during postzygotic development—anlage, new MICs, and pycnotic old MACs (see Fig. 1A). However, the following observations argue that actinomycin D-induced postzygotic abnormalities are the result of inhibition of gene expression from anlage and not from the pycnotic old MAC or new MICs. The old MAC is unlikely to be the source of this gene expression, since upon pycnosis it loses histones associated with active chromatin, becomes condensed and heterochromatic (Lin *et al.*, 1991), ceases incorporating [^{3}H]uridine (Wenkert and Allis, 1984), and its DNA is eventually degraded (Davis *et al.*, 1992). New MICs are also inactive during postzygotic development, as indicated by their failure to incorporate [^{3}H]-uridine (Wenkert and Allis, 1984).

The preceding evidence argues, by process of elimination, that the effects of actinomycin D are due to inhibition of gene expression in anlagen. This conclusion is

supported by direct evidence that anlagen are transcriptionally active during early MAC development. Anlagen acquire the expression-related acetylated histone H4 soon after they first appear (Lin *et al.*, 1991), and they begin accumulating [^{3}H]uridine as soon as conjugants reach the MACIIp stage at 6–7 hr (Wenkert and Allis, 1984). Thus, the most likely interpretation of our results is that expression of genes in the young anlagen is required to conduct postzygotic development. This in turn supports our earlier suggestion that the phenotypes of nullisomic conjugants also are caused by absence of such genes in the young anlagen (Ward *et al.*, 1995).

Timing of Gene Expression from the Developing MAC

Pair separation, old MAC resorption, and new MIC elimination each require gene expression at least ~1.5–2 hr before each event is completed (Fig. 3). Mayo and Orias (1986) showed that the time of induction of galactokinase enzyme production by conjugating cells could be delayed only if actinomycin D was added at least ~1.5–2 hr before onset of enzyme activity. Thus, similar actinomycin D lags are seen for simple enzyme (galactokinase) expression and the completion of cytological conjugation events, suggesting that gene products involved in pair separation, old MAC resorption, and MIC elimination complete their tasks soon after they are translated. Note that the time lag between the loss of drug effectiveness and execution of the step in each case represents the time between the expression of the latest gene required in the process and the time that the event is completed.

Pair separation is the earliest cytological event affected by actinomycin D, and its completion requires transcription of anlage-encoded information no later than ~9.5 hr during conjugation. To our knowledge this is the earliest demonstrated requirement for the postzygotic genome in *Tetrahymena.* This early expression precedes bulk DNA rearrangements by at least 3 hr. It has been suggested that germline-limited sequences enforce genome silencing in *Tetrahymena* (e.g., Katoh *et al.*, 1993; Yao *et al.*, 1984; but see Bannon *et al.*, 1984; and Pederson *et al.*, 1984). Our results cast further doubt on this notion since postzygotic gene expression precedes wholesale genome reorganization; however, it is possible that rearrangements occur in these critical postzygotic genes, but before the bulk of the rearrangements.

Apparent Multistep Processes

Actinomycin D apparently can interrupt pair separation, old MAC resorption, and MIC elimination at intermediate stages, suggesting that multiple episodes of transcription are required to complete each of these processes.

Recall that pair separation is blocked when actinomycin D is added early in the MACIIp stage. In contrast, drug addition ~2–3 hr prior to the time most pairs separate usually causes aberrant pair fusion. Note that this coincides with the time when actinomycin D begins to lose its effectiveness in inhibiting pair separation (Fig. 3A). Thus pair fusion results when cells have nearly finished expressing the latest gene(s) required for pair separation. Considering these observations, pair fusion could potentially be a by-product of an abortive attempt at pair separation in conjugants that have either not expressed all gene(s) required for pair separation or not expressed enough of a necessary gene product, before actinomycin D addition. Alternatively, pair fusion could be a consequence of anlage DNA loss and lethality (Fig. 4).

Resorption of the old MAC is a protracted process (Davis *et al.*, 1992) and is interruptable by actinomycin D addition throughout its course: for example, the old MACs in Fig. 2C are smaller and fainter than those in Fig. 2A (actinomycin D additions at 10 and 7 hr, respectively). Thus, complete old MAC resorption, like pair separation, appears to require multiple episodes of transcription, suggesting that multiple steps are required. In each case, these steps could be different or, especially in the case of old MAC resorption, could be reiterations of one step.

In at least one instance MIC elimination apparently was blocked at an intermediate stage (Fig. 2F). A potential explanation for this rare cell might be that it was just beginning to transcribe a necessary gene when actinomycin D was added, leaving the cell with a small dose of mRNA sufficient to direct the process at a very slow rate. Alternatively, like pair separation and old MAC resorption, MIC elimination could require multiple transcription-directed steps. Either way, the infrequency of such cases (relative to pair fusion and old MAC resorption failure) suggests that gene expression required for MIC elimination occurs within a short interval of time compared to pair separation and old MAC resorption.

While anlage DNA loss is not a normal consequence of postzygotic development, as the previous processes are, it apparently can be blocked at intermediate stages (see the example in Fig. 2F). As with the previously described processes, incomplete anlage DNA loss may indicate that some aspect of normal anlage development requires multiple episodes of gene expression (see below), which when inhibited by actinomycin D at different stages of development results in various degrees of anlage DNA loss.

Possible Role for Postzygotic Gene Expression in Anlage DNA Rearrangements

Ward *et al.* (1995) proposed that zygotically expressed genes are involved in anlage genome rearrangements, based on the epistatic interactions of nullisomic deficiencies influencing anlage fate. Deficiencies that block anlagen development early in the MACIIp stage are epistatic to those that result in aberrant anlage DNA loss, leading to the hypothesis that some genes are required to initiate chromosome breakage or DNA deletion events, while others are required for their completion. Thus, when nullisomic conjugants are deficient for genes required to initiate rearrangements, the anlage fail to proceed in development

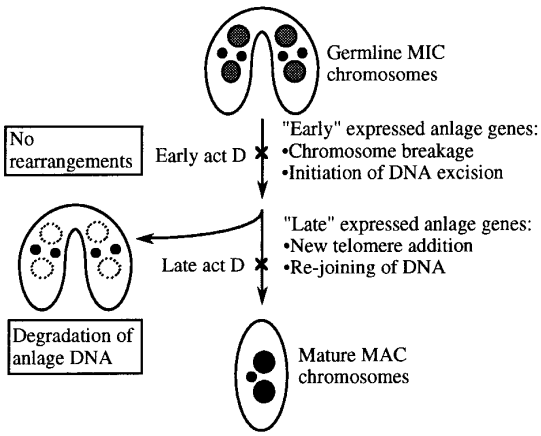


FIG. 5. Hypothetical role of anlage-expressed genes in anlage chromosome rearrangements. Germline chromosomes are extensively reorganized during MAC development to form MAC chromosomes (see review by Yao, 1989). The existence of anlage-expressed genes involved in DNA rearrangements are posited to explain the epistatic anlage phenotypes of nullisomic conjugants (Ward *et al.*, 1995), and phenocopies induced by actinomycin D in euploid conjugants (see text). Loss of ''early'' gene expression, either by treatments with actinomycin D initiated early in postzygotic development or by their deletion in nullisomic conjugants, blocks conjugants from initiating genome rearrangements. Possibly, these genes encode proteins that are involved in chromosome breakage or initiation of DNA excision events that lead to broken chromosome ends. If early genes are expressed, but expression of ''late'' genes are disrupted, either by actinomycin D addition at intermediate times or by nullisomic deletion, anlage DNA is degraded, because these late genes encode functions required to heal broken chromosome ends or to rejoin MAC-destined flanks of excision sequences. Symbols representing nuclei are as in Fig. 1.

and their DNA remains intact. However, when cells are deficient for genes required for completion of these events, they can begin DNA rearrangements and generate DNA breaks that cannot be ''healed,'' a condition that eventually can lead to anlage DNA loss.

The actinomycin D results presented here provide kinetic support for the epistasis model, as summarized in Fig. 5. If actinomycin D is added early, rearrangements do not begin, but cells can complete conjugation normally once the drug is removed. If actinomycin D is added after these ''early'' genes have been expressed, rearrangements are initiated, generating chromosome breaks (at chromosome breakage or DNA deletion sites), but the cell is unable to express ''late'' genes necessary to heal these breaks (i.e., by telomere addition or DNA rejoining), thus leading to eventual anlage DNA degradation and lethality.

The onset of lethality induction and DNA loss at 10 hr (Fig. 4) precedes by ~2 hr the onset of anlage DNA rearrangements, which normally begin at ~12 hr (Austerberry *et al.*, 1984; Yokoyama and Yao, 1982). Recall that a similar ~2-hr lag is observed for other events in conjugation and is attributed, in part, to the time between transcription of these genes and the completion of events they control. This observation supports the idea that DNA loss and inviability may be the result of inhibition of gene expression required for the completion of anlage DNA rearrangements.

The results discussed here demonstrate a major role of the postzygotic genome (anlage) in postzygotic development. The evidence also suggests that anlage-expressed genes may be involved in genome rearrangements. Given the evidence for the existence of such genes, it should be possible to isolate lethal mutations in these postzygotic genes and begin a more detailed genetic analysis of postzygotic development and genome reorganization in *Tetrahymena.*

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